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Acylating Phencyclidines Irreversibly Enhance Brain Calcium Antagonist Binding

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BOLGER, G. T., M. F. RAFFERTY, B. A. WEISSMAN, K. C. RICE AND P. SKOLNICK. *Acylating phencyclidines irreversibly enhance brain calcium antagonist binding.* PHARMACOL BIOCHEM BEHAV **25**(1) 51–57, 1986.— Phencyclidine was previously shown to allosterically increase the apparent affinity of the dihydropyridine ([^{3}H]nitrendipine) calcium antagonist binding site in a lysed synaptosomal membrane preparation of rat forebrain. Treatment of a similar preparation of mouse forebrain with 4-isothiocyanato-1-(1-phenylcyclohexyl) piperidine (FOURPHIT), an acylating phencyclidine derivative, resulted in a concentration dependent (0.1–10 μM), irreversible, increase in the apparent affinity of [^{3}H]nitrendipine in contrast to the effects of phencyclidine which were reversible. The FOURPHIT isomer, 1-[1-(3-isothiocyanatophenyl) cyclohexyl] piperidine (METAPHIT), (10 μM) also irreversibly increased the apparent affinity of [^{3}H]nitrendipine, but was much less efficacious than FOURPHIT. Phencyclidine blocked the irreversible increase in the apparent affinity of [^{3}H]nitrendipine produced by FOURPHIT. The interactions of multivalent cations and the calcium antagonist diltiazem with the [^{3}H]nitrendipine binding site were altered following treatment of membranes with FOURPHIT. These studies suggest that FOURPHIT irreversibly interacts with the same sites as PCP, and thus may be a useful tool with which to further probe both the behavioral and biochemical interactions between phencyclidine and the dihydropyridine calcium antagonist binding site.

Nitrendipine Dihydropyridine Calcium antagonists Calcium channels Acylating phencyclidines

DIHYDROPYRIDINE calcium antagonists (DHPCA) are potent inhibitors of calcium currents in peripheral tissues [14, 31, 32]. High affinity binding sites for DHPCA have been characterized in both peripheral tissues [1, 2, 15, 28, 30, 33] and brain [19, 25, 26, 28]. In contrast to peripheral tissues (where the DHPCA binding site functions as a calcium channel regulator [34]), the role of these sites in the central nervous system (CNS) is still unclear. Nonetheless, recent biochemical [16, 22, 35] and behavioral [3, 6, 12, 21, 28] evidence clearly suggests that DHPCA binding sites in brain may play an important neuromodulatory role in the CNS.

We recently reported [4,5] that the psychotomimetic phencyclidine (PCP) [9,17] and several related derivatives increased the apparent affinity of [^{3}H]nitrendipine in rat and mouse brain [4,5]. The enhancement of [^{3}H]nitrendipine binding by PCP was via an allosteric mechanism and displayed structural dependence, brain region specificity, a possible modulation by endogenous tissue factors, and was potently inhibited by Ca^{2+}. Neither the local anesthetic properties of PCP [4,8] nor an interaction of PCP with its high affinity binding site in brain [4,27] could account for this effect on [^{3}H]nitrendipine binding. These findings suggest that the DHPCA binding site in the CNS might represent a pharmacologically relevant locus of action for PCP [4,5].

We now report that the acylating PCP derivatives 4-isothiocyanato-1-(1-phenylcyclohexyl) piperidine (FOURPHIT) and 1-[1-(3-isothiocyanatophenyl) cyclohexyl) piperidine (METAPHIT) (Fig. 1) (the isomer characterized as an irreversible inhibitor of the [^{3}H]PCP binding site in rat brain [27]), can irreversibly increase the apparent affinity of [^{3}H]nitrendipine for DHPCA binding sites in a lysed synaptosomal membrane preparation of mouse forebrain; FOURPHIT being more efficacious than METAPHIT. The pharmacologic profile of FOURPHIT's actions suggests that it is interacting with the same site(s) as PCP to affect [^{3}H]nitrendipine binding. Furthermore, through the use of FOURPHIT, it was determined that PCP interacts with two sites to alter [^{3}H]nitrendipine binding.

METHOD

Tissue Preparation

Male NIH mice (ICR, Veterinary Resources Branch, National Institutes of Health, Bethesda, MD: 18–22 g) were

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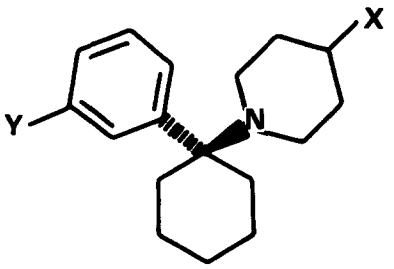

PCP : X = Y = – H
METAPHIT : X = – H, Y = – NCS
FOURPHIT : X = – NCS, Y = – H

FIG. 1. Structures of PCP, FOURPHIT and METAPHIT.

used in all experiments. A lysed synaptosomal enriched membrane fraction from mouse forebrain was prepared according to Whittaker [33]. Mice were killed by decapitation, their brains quickly removed and placed into ice cold 0.32 M sucrose. The forebrain was isolated by making an oblique cut from the superior colliculus on the dorsal surface to the mammilary bodies on the ventral surface. Tissue was homogenized in 20 vol 0.32 M sucrose using ten up and down strokes of a glass-teflon pestle (clearance 0.13–0.18 mm), motor driven homogenizer. The homogenate was centrifuged at 1,100 × g for 10 min, the pellet discarded and the supernatant recentrifuged at 24,000 × g for 20 min. The resulting pellet from the centrifugation was lysed by resuspending in 70 vol of 5 mM Tris HCl, pH 7.4 using a polytron (Brinkman, 5 sec, speed setting 6–7). This membrane suspension was used for radioligand binding and drug treatments.

Incubation of Membranes With PCP Alkylators

FOURPHIT or METAPHIT (as the hydrochloride salts) were taken up in distilled water at 22°C. New solutions were prepared for each experiment. Unless otherwise specified (in table and figure legends), all incubations were performed in 5 mM Tris HCl buffer, pH 7.4. Aliquots of membrane suspension were incubated with alkylating agents (0.1–100 μM) at 4°C for 30 min. For control conditions, membrane aliquots were taken from the same preparation and incubated at 4°C for the same time period; subsequent treatment being identical to those membranes incubated with alkylator. Excess alkylating agent was removed by centrifugation of membranes at 24,000 × g for 10 min, followed by resuspension of the pellet in 70 vol 5 mM Tris HCl. Unless otherwise stated (in table and figure legends), membranes were centrifuged and resuspended in buffer one more time as described above and subsequently used for radioligand binding.

[^{3}H]Nitrendipine Binding

[^{3}H]Nitrendipine binding was assayed in a total volume of 2 ml in borosilicate glass tubes under subdued fluorescent light. The assay volume consisted of 0.9 ml of 5 mM Tris HCl buffer containing drug(s), 0.1 ml [^{3}H]nitrendipine and 1 ml of membrane suspension (0.3–0.5 mg protein). Incubations at 25°C were initiated by addition of tissue and terminated after 1 hr by rapid filtration through Whatman GF/B glass fibre filters followed by 2×5 ml washes with ice cold 5 mM Tris buffer using a Brandel Cell Harvester (Model 24R, Brandel Co., Gaithersburg, MD). The filters were placed in 8 ml of Beckman Ready-Solv, MP (Beckman Instruments, Inc., Fullerton, CA) and counted by liquid scintillation spectrophotometry (Beckman Model LS 5800). Specific [^{3}H]nitrendipine bound was defined as the difference between [^{3}H]nitrendipine binding in the presence and absence of 10^{-6} M nifedipine.

TABLE 1

THE EFFECTS OF FOURPHIT AND METAPHIT ON [^{3}H]NITRENDIPINE BINDING

Condition*	Kd (pM)	Bmax (fmol/mg protein)
Control (no drug)	182.5 ± 17.2	223.9 ± 40.9
PCP 10 μM	187.9 ± 17.7	228.3 ± 41.9
FOURPHIT 10 μM	80.4 ± 8.8†	198.8 ± 27.2
METAPHIT 10 μM	143.7 ± 14.6‡	218.0 ± 36.2
PCP 100 μM + FOURPHIT 10 μM	156.2 ± 14.7‡§	232.8 ± 42.6

[^{3}H]Nitrendipine binding to mouse forebrain membranes was measured in the concentration range of 30–1,200 pM. *Membranes were pretreated with PCP, FOURPHIT or METAPHIT (see the Method section). The results are presented as the mean ± S.E.M. of four experiments. Significantly different from control; †$p<0.005$; ‡$p<0.05$; §significantly different from FOURPHIT alone $p<0.005$; unpaired t-test.

Protein Determination

Protein was determined by the Miller modification [23] of the Lowry assay [19] using bovine serum albumin as a standard.

Materials

[^{3}H]Nitrendipine (70–80 Ci/mmol) was obtained from New England Nuclear. The commercial preparation was diluted 1-fold with absolute ethanol and stored in the dark at −20°C. Subsequent dilutions for use in binding experiments were prepared in 5 mM Tris HCl, pH 7.4, and kept at 4° in aluminum foil wrapped containers.

FOURPHIT was prepared starting with the known, 1-(1-phenylcyclohexyl)-4-piperidone [13]. The ketone was converted to the oxime by treatment with 1 mole equivalent of hydroxylamine hydrochloride in ethanol and refluxed for 1 hr. The crude oxime was reduced by careful addition of sodium metal in small pieces (approximately 20 mole equivalents) to a refluxing mixture of the oxime in absolute ethanol. The resulting amine was crystallized as the bis (hydrochloride) monohydrate salt, m.p. 181–184°C. Treatment of the free base of the amine with thiophosgene (1.5 mole equivalents), in a chloroform-aqueous sodium bicarbonate medium under standard conditions used elsewhere [7] yielded FOURPHIT, which was isolated as the hydrochloride salt, m.p. 176–180°C (crystallized from isopropyl alcohol/isopropyl ether mixture). Spectra and combustion analysis of FOURPHIT and the reported intermediates were in accord with the assigned structures. METAPHIT was prepared as previously described [27]. Phencyclidine hydrochloride was obtained from the NIDA, Rockville, MD. N-propyl-1-phenylcyclohexylamine (N-propyl-PCA) hydro-

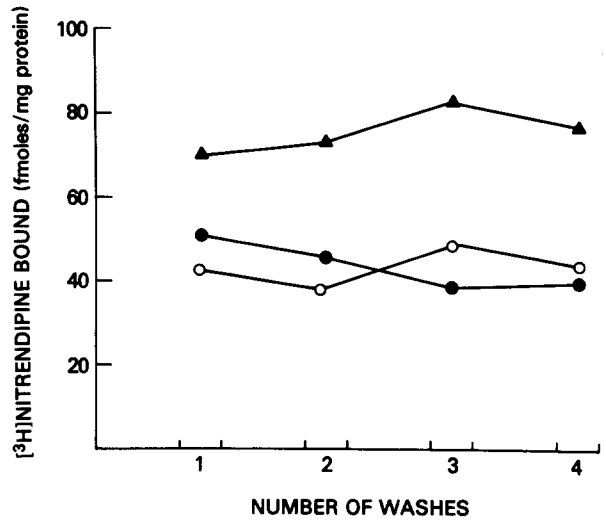

FIG. 2. Effects of FOURPHIT and PCP on [^{3}H]nitrendipine binding following extensive washing. [^{3}H]Nitrendipine binding (120 pM) was investigated in mouse forebrain membranes that were untreated (●), pretreated with PCP (○) 10 μM, or pretreated with FOURPHIT (▲) 10 μM and then subjected to the number of washes (with 5 mM Tris HCl) indicated. The results are the mean of two experiments.

chloride was kindly provided by Warner-Lambert Co., Ann Arbor, MI. Diltiazem and verapamil were obtained from Marion Laboratories, Kansas City, MO and Knoll, A. G., West Germany, respectively. Tris base and all inorganic salts were obtained from standard commercial sources.

RESULTS

Effects of FOURPHIT and METAPHIT on [^{3}H]Nitrendipine Binding

Consistent with previous findings [4], coincubation of PCP (10 μM) with [^{3}H]nitrendipine in a lysed (synaptosomal membrane preparation of mouse forebrain resulted in an increase in the apparent affinity (decreased Kd) of [^{3}H]nitrendipine (Kd and Bmax values, control 164.4 pM, 173.4 fmol/mg protein; + PCP (10 μM) 89.8 pM, 183.2 fmol/mg protein respectively). Treatment of membranes with FOURPHIT (10 μM) followed by successive washes with 5 mM Tris HCl, resulted in a significant decrease (56%) in the Kd of [^{3}H]nitrendipine (Table 1). Identical treatment of membranes with METAPHIT (10 μM) resulted in a smaller, but statistically significant decrease (20%) in the Kd of [^{3}H]nitrendipine (Table 1). However, following successive washes of PCP treated membranes, the Kd of [^{3}H]nitrendipine was not significantly different from that prior to PCP treatment (Table 1). Coincubation of either FOURPHIT (10 μM) or METAPHIT (10 μM) with [^{3}H]nitrendipine decreased the Kd of this ligand to the same extent as did coincubation with PCP (10 μM).

The reversible nature of PCP versus the irreversible nature of FOURPHIT was further evaluated by extensive washing of pretreated membranes with 5 mM Tris HCl. While the increase in [^{3}H]nitrendipine binding mediated by FOURPHIT (10 μM) pretreatment could not be reversed following four washes (Fig. 2), that mediated by PCP was reversed >90% following a single wash (Fig. 2).

The interaction of FOURPHIT at the [^{3}H]nitrendipine

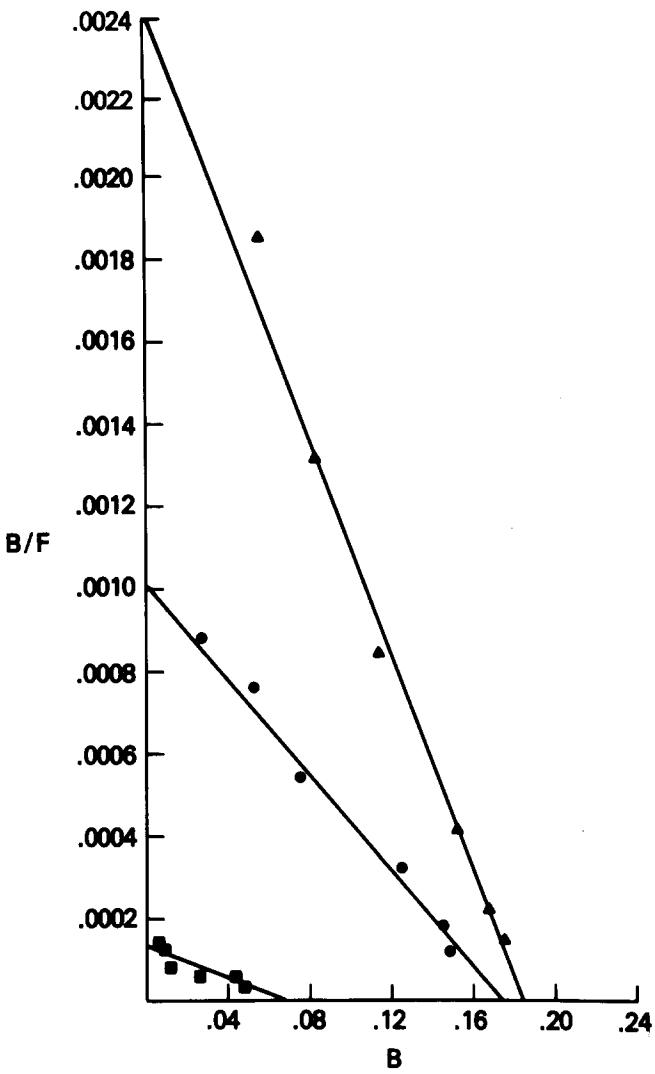

FIG. 3. Scatchard analysis of the effects of FOURPHIT on [^{3}H]nitrendipine binding. [^{3}H]Nitrendipine binding to mouse forebrain membranes was investigated over the concentration range of 35–1,200 pM, in untreated, and membranes treated with FOURPHIT 10 μM, and FOURPHIT 100 μM. The Kd and Bmax values were for untreated (●) 164.36 pM 173.40 fmol/mg protein; FOURPHIT 10 μM (▲) 74.16 pM, 184.36 fmol/mg protein and FOURPHIT 100 μM (■) 506.04 pM, 68.32 fmol/mg protein respectively. B is bound (fmol/mg protein) and F is free (FM). The results are the mean of 2 experiments.

binding site was complete within 15 min. This time scale is consistent with that observed for the specific alkylation of the neuronal [^{3}H]PCP binding site by METAPHIT [25]. While FOURPHIT (10 μM) decreased the Kd of [^{3}H]nitrendipine, a ten-fold higher concentration produced an increase (~3-fold) in the Kd and decrease (65%) in the Bmax of [^{3}H]nitrendipine (Fig. 3). This effect of FOURPHIT was irreversible, while that of PCP (100 μM) was completely reversible.

The possible interaction of FOURPHIT and PCP at the same site(s) to affect [^{3}H]nitrendipine binding was investigated. Coincubation of 100 μM PCP with 10 μM FOURPHIT resulted in a significant inhibition of the irreversible decrease

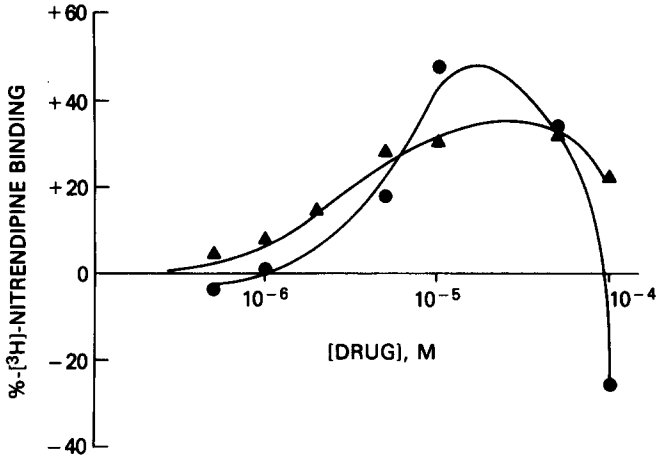

FIG. 4. Enhancement of [^{3}H]nitrendipine binding by FOURPHIT and PCP. Enhancement of [^{3}H]nitrendipine (165 pM) binding to mouse forebrain membranes was investigated during coincubation with PCP (▲) or following treatment with FOURPHIT (●) over the concentration range shown. The results are the mean of 2 experiments.

in the Kd of [^{3}H]nitrendipine mediated by FOURPHIT (Table 1). FOURPHIT irreversibly increased [^{3}H]nitrendipine binding with a concentration dependence similar to that for coincubation of PCP with [^{3}H]nitrendipine (Fig. 4). Following treatment of membranes with FOURPHIT (10 μM), no further change in [^{3}H]nitrendipine binding was observed in the presence of 10 μM PCP (Table 2). However, a significant further increase was observed following METAPHIT (10 μM) treatment (Table 2). In addition, following FOURPHIT treatment, both PCP (Fig. 5A) and N-propyl-PCA (Fig. 5B) produced only an inhibition of [^{3}H]nitrendipine binding.

Effect of FOURPHIT on the Interaction of Cations and Calcium Channel Antagonists With [^{3}H)Nitrendipine Binding

Pretreatment of mouse brain membranes with FOURPHIT (10 μM) markedly altered the interactions of cations with [^{3}H]nitrendipine binding. Na^+, K^+, Ca^{2+}, and Mg^{2+} increased, while Ni^{2+} decreased [^{3}H]nitrendipine binding (Table 3). Following FOURPHIT the effects of Ni^{2+}, Ca^{2+}, and Mg^{2+} were inhibited, while those of Na^+ and K^+ were not (Table 3). FOURPHIT decreased the potency of La^{3+} to inhibit [^{3}H]nitrendipine binding (Table 4). With respect to organic calcium antagonists, FOURPHIT did not change the potency of either nifedipine or verapamil to inhibit [^{3}H]nitrendipine binding. In agreement with previous findings [2,17], diltiazem both inhibited (10^{-9}–10^{-6} M) and increased (10^{-6}–10^{-5} M) [^{3}H]nitrendipine binding. Following FOURPHIT (10 μM) treatment, diltiazem (10^{-9}–10^{-5} M) produced only an inhibition of [^{3}H]nitrendipine binding.

DISCUSSION

We have recently demonstrated that the psychotomimetic PCP and a number of pharmacologically related compounds enhanced [^{3}H]nitrendipine binding to both rat and mouse brain membranes by allosterically increasing the affinity of the DHPCA binding site [4,5]. This effect demonstrated

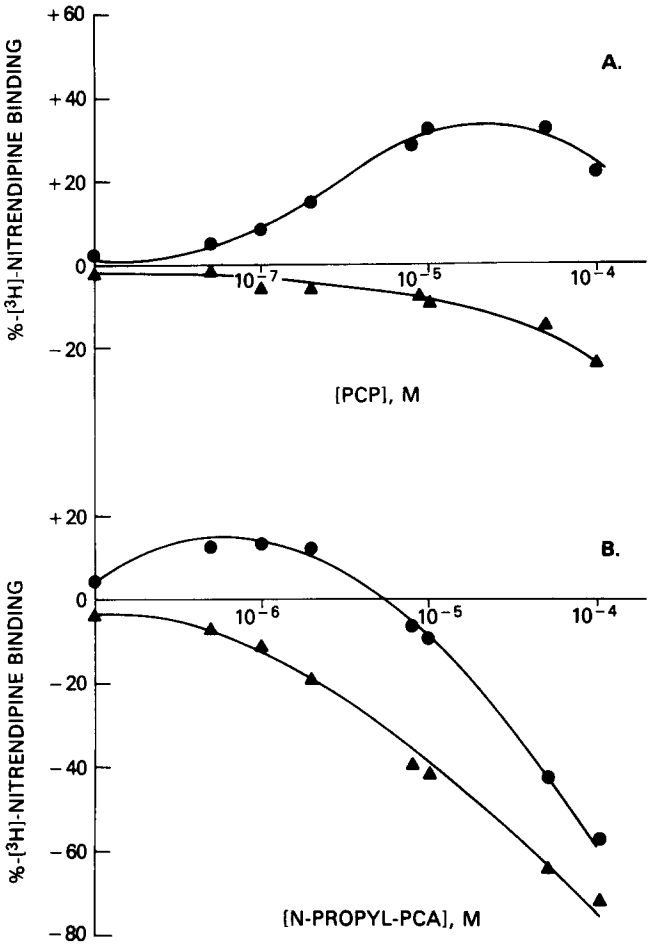

FIG. 5. The effects of FOURPHIT on enhancement of [^{3}H]nitrendipine binding by PCP and N-propyl-PCA. The enhancement of [^{3}H]nitrendipine binding to mouse brain membranes by (A) PCP and (B) N-propyl-PCA was investigated in untreated (●) membranes and those treated with FOURPHIT 10 μM (▲). The results are expressed as the percent of [^{3}H]nitrendipine binding in the absence of PCP or N-propyl-PCA and are the mean of 2 experiments.

brain region selectivity, inhibition by divalent cations, a possible regulation by endogenous tissue factors, and appears unrelated to the local anesthetic properties of PCP or the high affinity PCP binding site.

Insertion of an isothiocyanate moiety on the 'meta' position of the aryl ring (METAPHIT) or the 4'-position of the piperidine ring (FOURPHIT) of PCP, produced compounds that irreversibly increased [^{3}H]nitrendipine binding. In contrast, increases in [^{3}H]nitrendipine binding mediated by PCP were completely reversible. Structural selectivity was evident, since FOURPHIT was more efficacious than METAPHIT at enhancing [^{3}H]nitrendipine binding. Differences in potency between these compounds could not account for this observation, since higher concentrations of METAPHIT inhibited [^{3}H]nitrendipine binding (results not shown). This observation supports previous findings demonstrating that compounds pharmacologically related to PCP differed in their efficacies, but not their potencies to enhance [^{3}H]nitrendipine binding [5], consistent with an allosteric mechanism of action. The reactivity of FOURPHIT at the

TABLE 2

ENHANCEMENT OF [^{3}H]NITRENDIPINE BINDING BY PCP FOLLOWING TREATMENT WITH FOURPHIT AND METAPHIT

Condition*	[^{3}H]Nitrendipine Bound (fmol/mg protein)
Control (no drug)	68.4 ± 1.2
PCP 10 μM	91.7 ± 1.7†
FOURPHIT treated	104.7 ± 1.6†
FOURPHIT treated + PCP 10 μM	97.9 ± 1.9†
METAPHIT treated	76.9 ± 1.7†
METAPHIT treated + PCP 10 μM	92.7 ± 7.4†‡

[^{3}H]Nitrendipine (120 pM) binding was measured in mouse forebrain membranes. *Membranes were pretreated with either FOURPHIT or METAPHIT (10 μM). PCP was coincubated with [^{3}H]nitrendipine. The results are presented as the mean ± S.E.M. of five to six experiments. †Significantly different from control $p<0.005$; ‡significantly different from METAPHIT alone $p<0.05$, unpaired t-test.

TABLE 3

THE EFFECT OF FOURPHIT ON THE CATION SENSITIVITY OF [^{3}H]NITRENDIPINE BINDING

Cation	Concentration (mM)	% Control [^{3}H]Nitrendipine Binding	
		Untreated*	FOURPHIT treated*
Ca^{2+}	0.5	127 ± 1†	101 ± 4
Mg^{2+}	1.0	113 ± 2†	102 ± 5
Ni^{2+}	1.0	65 ± 5†	101 ± 3
Na^{+}	100.0	126 ± 2†	128 ± 1†
K^{+}	100.0	124 ± 3†	126 ± 7†

[^{3}H]Nitrendipine (120 pM) binding was measured in mouse forebrain membranes. *Membranes were untreated or treated with FOURPHIT 10 μM. The results are presented as ± S.E.M. of three experiments. †Significantly different from control $p<0.05$, unpaired t-test.

TABLE 4

THE EFFECT OF FOURPHIT ON THE POTENCY OF CALCIUM ANTAGONISTS FOR INHIBITION OF [^{3}H]NITRENDIPINE BINDING

Calcium Antagonist	Value*	Inhibition Constant (M)	
		Untreated†	FOURPHIT treated†
Nifedipine	Ki	$1.0 \pm 0.1 \times 10^{-9}$	$1.3 \pm 0.2 \times 10^{-9}$
Verapamil	IC_{50}	$2.1 \pm 0.7 \times 10^{-8}$	$2.3 \pm 0.6 \times 10^{-8}$
La^{3+}	IC_{50}	$7.1 \pm 0.4 \times 10^{-5}$	$1.3 \pm 0.1 \times 10^{-4}$*

[^{3}H]Nitrendipine binding (120 pM) was studied in a preparation of mouse forebrain. *IC_{50} values are presented for verapamil and La^{3+} due to the noncompetitive nature of their interaction with [^{3}H]nitrendipine [2]. †Membranes were untreated or treated with FOURPHIT 10μM. The results are presented as the mean ± S.E.M. of four experiments. *Significantly different from untreated, $p<0.05$, unpaired t-test.

DHPCA binding site suggests that the site of interaction for this drug contains a suitably located nucleophile capable of interacting with the isothiocyanate moiety [7,36].

The irreversible interaction of FOURPHIT with [^{3}H]nitrendipine binding was inhibited by coincubation with a ten-fold molar excess of PCP. This finding, coupled with the demonstration of a similar concentration dependence for FOURPHIT and PCP to increase [^{3}H]nitrendipine binding (and the inability of PCP to further increase [^{3}H]nitrendipine binding following treatment of membranes with FOURPHIT), is consistent with an interaction of these drugs at a common site. The observation that PCP could further increase [^{3}H]nitrendipine binding following treatment of membranes with METAPHIT (10 μM) suggests that the small enhancement of binding by this drug may arise as a consequence of its specific alkylation of a site other than that with which PCP interacts to increase DHPCA binding.

Treatment of membranes with 100 μM FOURPHIT resulted in a marked increase in the Kd and decrease in the Bmax of [^{3}H]nitrendipine binding. The differential effect of this concentration of FOURPHIT when compared to that at 10 μM can be most readily interpreted by two distinct sites of action, as has been proposed to explain the biphasic concentration dependent effects of PCP on [^{3}H]nitrendipine binding to rat brain membranes [4,5]. The observation that PCP or N-propyl-PCA produced only an inhibition of [^{3}H]nitrendipine binding following treatment of membranes with 10 μM FOURPHIT, also supports the notion that these drugs interact with two sites to alter [^{3}H]nitrendipine binding. The decrease in the Bmax of [^{3}H]nitrendipine produced by FOURPHIT suggests a direct action at the DHPCA binding site at high concentrations of this compound. Thus, with respect to PCP, two concentration dependent sites of action can be proposed: (1) in the concentration range 0.1–10 μM, to allosterically increase the apparent affinity of the [^{3}H]nitrendipine binding site and (2) at concentrations >10 μM, to inhibit [^{3}H]nitrendipine binding. A proposal of multiple sites of action for PCP is consistent with the biochemical heterogeny of PCP binding sites in the CNS [11,27].

FOURPHIT treatment markedly altered the specific interaction of metal cations (unpublished observations) with the [^{3}H]nitrendipine binding site in a 5 mM Tris HCl buffer. The effects of divalent cations (Ca^{2+}, Mn^{2+}) were completely inhibited while those of monovalent cations were not. These findings confirm earlier observations indicating that PCP interacts with a site that is involved in the divalent cation regulation of [^{3}H]nitrendipine binding [4]. FOURPHIT produced similar effects on the interactions of La^{3+}, verapamil and diltiazem with the [^{3}H]nitrendipine binding site as those reported for PCP in rat brain [5]. FOURPHIT did not increase the affinty of nifedipine for the DHPCA binding site in mouse brain, while PCP did increase the affinity of nifedipine for the DHPCA binding site in rat brain [5]. These findings may indicate that the structurally dependent PCP mediated increase in the affinity of DHPCAs for their binding sites in rat brain [5] is different for mouse brain.

Of the two acylating PCP derivatives investigated, FOURPHIT appears to covalently modify the allosteric site with which PCP interacts to increase the affinity of neuronal [^{3}H]nitrendipine binding. In addition, FOURPHIT irreversibly inhibited [^{3}H]nitrendipine binding potentially indicating a direct action for PCP at the DHPCA binding site.

Several lines of evidence [6, 24, 28] indicate that DHP calcium antagonists can modify PCP induced behaviors. Re-

cently, it was demonstrated behaviorally that METAPHIT could act as a less potent, long-acting, specific PCP-like agonist [17]; the behavioral effects of FOURPHIT presently unknown. In light of these findings and evidence for a biochemical interaction between PCP and behaviorally relevant DHPCA binding sites in neuronal tissue [4,5], FOURPHIT may prove to be a useful investigational tool with which to further elucidate the mechanism(s) involved in the biochemical and behavioral interactions between DHPCA and PCP.

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